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Effects of Photoperiod and Temperature on Egg-Laying Behavior in a Marine Mollusk, *Aplysia californica*

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Abstract. The primary purpose of these studies was to determine whether photoperiodic signals could influence seasonal egg-laying behavior in the marine mollusk, *Aplysia californica.* Egg-laying behavior was monitored from groups of animals that were collected at four times of year and maintained in different temperature and photoperiodic conditions in the laboratory. Animals that were obtained in autumn and kept in warm water laid eggs more frequently than those in cold water, regardless of photoperiod. Furthermore, animals maintained on short days and warm water laid eggs more frequently than those on long days and warm water. Animals in cold water showed little to no egg laying, and a photoperiodic response was not evident. Animals that were collected in either winter or spring and maintained in warm water showed little or no spontaneous egg laying throughout the study, regardless of photoperiod. As with the autumn animals, *Aplysia* individuals obtained in summer and kept on short days and warm water laid eggs more frequently than those kept on long days and warm water. These results provide the first evidence that the reproductive system of *A. californica* is responsive to photoperiod. Overall, the data suggest that warm water is permissive for egg laying, and that short days can further stimulate this behavior. However, there is a strong inhibition of spontaneous egg laying during the winter and spring, which neither warm water nor short photoperiod can overcome. The role of the eyes in mediating the photoperiodic response was also investigated. A control group of intact animals kept on short days laid eggs more frequently than those on long days, but this photoperiodic response was not evident in eyeless animals. These results suggest that the eyes play a role in mediating the effects of photoperiod on egg laying behavior.

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Introduction

Like many animals living in the temperate zone, the marine mollusk *Aplysia californica* breeds seasonally. Both field and laboratory observations indicate that this species is reproductively competent during the summer and autumn, and reproductively quiescent during the winter and spring (Strumwasser *et al.*, 1969; Audesirk, 1979; Berry, 1982). The onset of the breeding season is indicated by a significant increase in the incidence of copulation and egg laying (Strumwasser *et al.*, 1969; Audesirk, 1979), as well as increased synthesis of the hormone that controls egg laying (egg laying hormone; Berry, 1982).

Earlier work has shown that egg laying hormone, a peptide synthesized and secreted by the neuroendocrine bag cells, is responsible for triggering egg-laying behavior (Strumwasser *et al.*, 1969; Kupfermann, 1970; Arch, 1972; Dudek *et al.*, 1980; Stuart *et al.*, 1980; Chiu and Strumwasser, 1981; Blankenship *et al.*, 1983). Although much is known about the molecular biology of bag-cell peptides (Chiu *et al.*, 1979; Heller *et al.*, 1980; Scheller *et al.*, 1982; Mahon and Scheller, 1983) and about the electrophysiological properties of bag cells (Kupfermann and Kandel, 1970; Kaczmarek *et al.*, 1978, 1982; Kaczmarek and Strumwasser, 1981), the seasonal regulation of bag-cell activity and egg laying remains obscure. The general goal of this and future studies is to gain insight into the mechanisms underlying seasonal fluctuations in egg laying behavior and in reproductive neuroendocrine function.

The occurrence of reproductive activity at a particular time of year suggests the involvement of some environmental timing agent (*e.g.*, ambient temperature, photoperiod, food availability, specific nutritional cue). Previous studies in *Aplysia* have shown that warm water can stimulate egg laying, whereas cold temperatures inhibit this behavior (Berry, 1984; Pinsker and Parsons, 1985). Although the authors interpreted their results to suggest that changes in the rate of egg laying are solely dependent on seasonal cycles of temperature, the studies did not test for effects of other environmental variables, such as photoperiod.

The annual cycle of photoperiod is the most regular and predictable environmental factor, and is therefore used by a wide variety of temperate-zone species to time reproduction to the appropriate season (mammals: Turek and Campbell, 1979; birds: Rowan, 1926; reptiles: Licht, 1967; insects: Lees, 1966; terrestrial slugs: Sokolove *et al.*, 1984). *A. californica* are intertidal organisms, spending much of their time near the water surface (Audesirk, 1979); thus they would be exposed to annual changes in day length. *A. californica* might use photoperiodic information, as well as temperature cues, to synchronize reproduction to a particular time of year. The main goal of this study was to determine whether egg laying behavior can be influenced by photoperiodic signals.

Materials and Methods

General

Specimens of *Aplysia californica* (200–300 g) were captured off the coast of California (approximately 34°N latitude) by Alacrity Marine Supply, Redondo Beach, California. At the collection sites, the annual range in water temperature is from approximately 10 to 20°C (Dan Stark, Alacrity Marine Supply, pers. comm.), and the annual range in photoperiod is from 11 to 15.5 h light/day (includes 1 h civil twilight). Upon arrival in the laboratory, animals were maintained in temperature- and light-controlled seawater tanks (475 liters; light intensity at water surface was 700 lux as measured with a photographic light meter). Water was recirculated through undergravel filters within the tanks. Treatment groups (initially, 12 animals per group; 0–3 animals/group died during the course of the studies) were maintained in separate tanks, and all animals were kept in single, perforated plastic buckets (20 cm in diameter) so that each individual could be monitored throughout the studies.

To document the egg laying capability (*i.e.*, reproductive maturity) of each animal, atrial gland extract was injected into the hemolymph of all animals upon arrival in the laboratory (Nagle *et al.*, 1985). Animals with a mature reproductive system will lay eggs in response to atrial gland extract, while immature animals will not lay eggs. An *Aplysia* that did not lay eggs spontaneously during the course of the studies was again treated with atrial gland extract at the end of each study to assess maturity. Only those animals that were reproductively mature by the end of the studies were included in the analysis. Animals were fed a combination of Romaine lettuce and dried seaweed (Msubi Nori, Japan Food Corp.) daily. Egg masses were recorded daily from individual buckets. Because *Aplysia* lays eggs at a maximal rate of once per day and does not consume its own eggs (unpub. obs.), the presence or absence of an egg mass is an excellent indication of whether an animal exhibited egg laying behavior on any given day.

The effects of photoperiod on egg laying behavior were determined as follows. Specimens of *Aplysia* were collected and shipped to our seawater facilities at four different times of year. Animals that arrived in the early AUTUMN 1988 (Sept. 22) were all reproductively mature at the beginning of the study and were divided into four treatment groups. *Aplysia* individuals were kept either on short days (8 h light/day) or on long days (16 h light/day); animals on these two photoperiods were further divided and maintained either in warm (20°C) or in cold (15°C) water. Thus the combined effects of photoperiod and water temperature on egg laying could be investigated. Animals maintained in cold water rarely, if ever, layed eggs, so we dropped the cold-water group from the remaining studies. *Aplysia* individuals that arrived in the early WINTER 1989 (Jan. 3) and the early SPRING 1989 (Mar. 31) were reproductively immature at the beginning of the studies; but they had all reached maturity by the end of the experiments. In these two studies, all animals were maintained in warm water and kept either on short or on long days. *Aplysia* individuals that arrived in the early SUMMER 1989 (June 23) were reproductively mature at the beginning of the study and were maintained in warm water and kept either on short or on long days.

The role of the eyes in mediating the effects of photoperiod on egg laying was investigated with specimens of *Aplysia* that were brought to the laboratory in the late SUMMER 1990 (Aug. 7) and maintained in warm water and 14.25 h light/day (photoperiod in mid-August at 34°N latitude) for three days. All of these animals were immobilized with $MgCl_2$ (injected into hemolymph); half of them were bilaterally enucleated, and the other half served as intact controls. Following surgery, animals were further divided and kept either on short (8 h light/day) or on long days (16 h light/day), making a total of four treatment groups.

Analysis of data

Differences in egg laying between treatment groups were assessed by Chi-square analysis. Values were significantly different if $P < 0.05$.

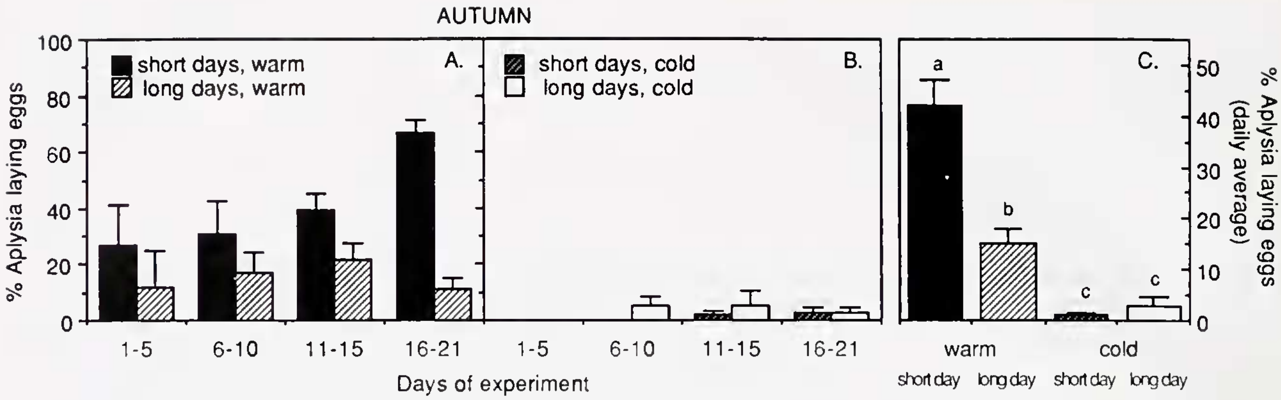

Figure 1. Percent of *Aplysia* individuals laying eggs during the early AUTUMN 1988. Panel A: Animals were kept in warm (20°C) water and either short (8 h light/day) or long (16 h light/day) days. Data were averaged (+sem) into 5-day bins. Panel B: Animals were kept in cold (15°C) water and either short (8 h light/day) or long (16 h light/day) days. Data are presented as in panel A. Panel C: Data from the 4 groups are presented as the percent of animals laying eggs each day, averaged (+sem) over the entire 21-day study. Different letters indicate values are significantly different (at least $P < 0.05$).

Results

Photoperiodic effects on egg laying

Overall, photoperiod and temperature can both affect the frequency of egg laying. In the AUTUMN, *Aplysia* individuals kept in warm water laid eggs more frequently than those kept in cold water (Fig. 1). Furthermore, animals maintained on short days and warm water laid eggs more frequently than those kept on long days and warm water. However, in the WINTER (Fig. 2) and in the SPRING (Fig. 3), egg laying frequency overall was suppressed in all groups (even though animals were reproductively mature by the end of the studies; see Materials and Methods), and there was no apparent effect of photoperiod on egg laying. In the SUMMER (Fig. 4), we once again observed the emergence of a photoperiodic effect: *Aplysia* maintained on short days and warm water laid eggs more frequently than those kept on long days and warm water. This photoperiodic response in the summer was not as robust as that seen during the previous autumn (compare Figs. 1c and 4b).

Photoperiodic effects in intact vs. eyeless animals

The eyes appear to play a role in transducing photoperiodic information to the reproductive axis responsible for regulating egg laying (Fig. 5). Once again, control animals kept in short days and warm water laid eggs more frequently than those kept on long days and warm water.

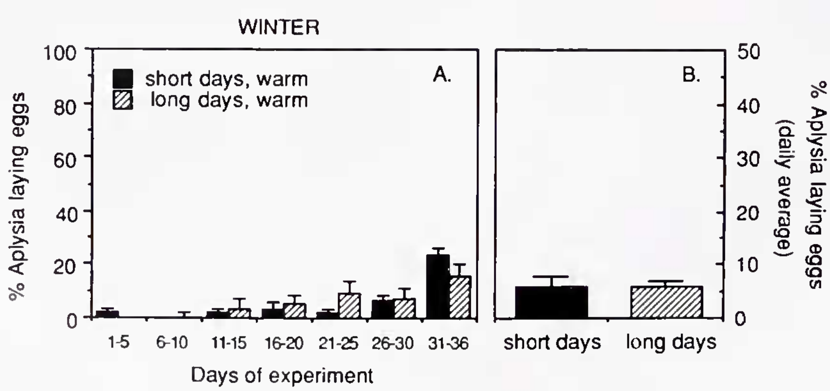

Figure 2. Percent of *Aplysia* individuals laying eggs during the early WINTER 1989. Animals were kept in warm (20°C) water and either short (8 h light/day) or long (16 h light/day) days. Panel A: Data were averaged (+sem) into 5-day bins. Panel B: Data are presented as the percent of animals laying eggs each day, averaged (+sem) over the entire study. There was no significant difference between the values of the two groups.

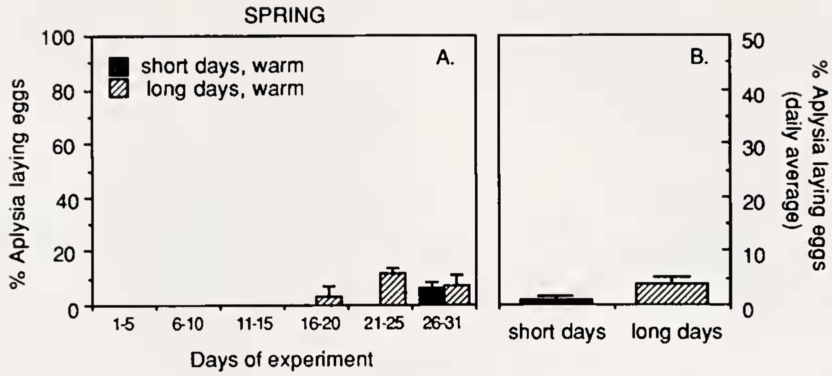

Figure 3. Percent of *Aplysia* individuals laying eggs during the early SPRING 1989. Data are presented as in Figure 2.

On the other hand, there was no significant difference in the frequency of egg laying between the two eyeless groups. Although the photoperiodic response in the intact control group was significant, it was not nearly as robust as that seen in a previous study (see Fig. 1).

Discussion

This study provides the first evidence that the reproductive system of *Aplysia* is responsive to photoperiodic signals. The results suggest that both photoperiod and temperature can influence the seasonal rhythm of egg laying. Specifically, warm temperature is permissive for the expression of the stimulatory effects of short days. Studies in another poikilotherm, the lizard *Anolis carolinensis,* have also documented that the reproductive response to stimulatory day lengths is evident in warm, but not cool, temperatures (Licht, 1967). In addition, recent work in the edible snail *Helix pomatia* has shown that egg-laying behavior is regulated by both photoperiod and temperature cues (Gomot, 1990). In the wild, the reproductive activity of *Aplysia californica* peaks in late summer-autumn (Strumwasser *et al.,* 1969; Audesirk, 1979; Berry, 1982). At this time of year, water temperature is reaching a maximum off the coast of California, and day length is decreasing. Our findings that warm water and short days stimulate egg laying are therefore consistent with the behavior of the animal in its natural environment.

Animals brought to the laboratory in the winter and spring layed eggs infrequently, if at all, regardless of environmental treatment. That is, an average of less than 10% of the winter and spring animals laid eggs on any given day during the course of the two studies—even under stimulatory conditions of short days and warm water. Although these animals were reproductively immature at the onset, towards the end of the studies they had reached maturity and were capable of laying eggs following hormonal stimulation (see Materials and Methods). Therefore, ovotesticular function was most likely not the limiting factor in these studies (however, we do not know when during the studies animals attained reproductive maturity).

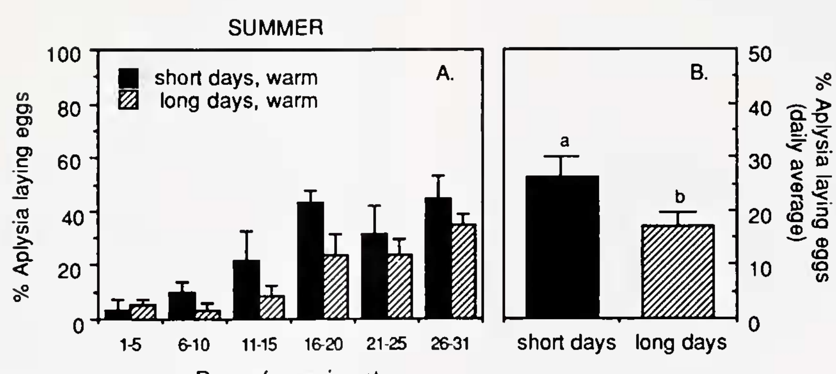

Figure 4. Percent of *Aplysia* individuals laying eggs during the early SUMMER 1989. Data are presented as in Figure 2. In Panel B, different letters indicate values are significantly different ($P < 0.05$).

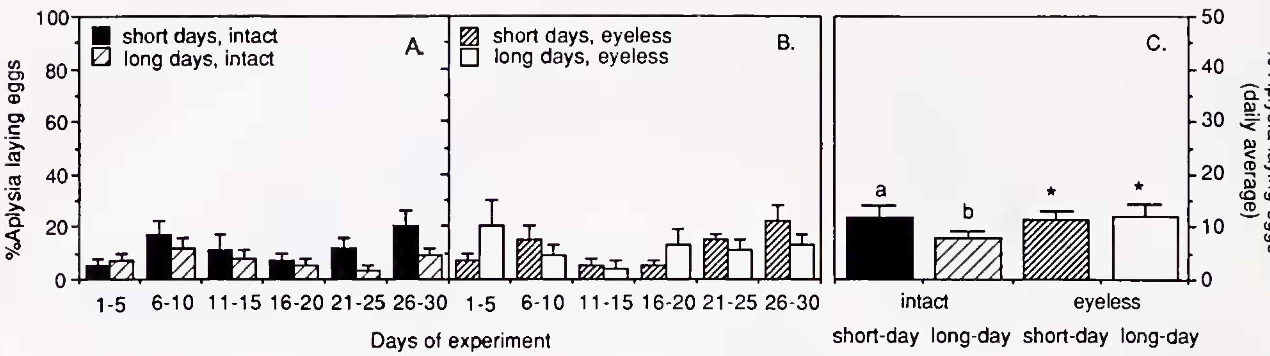

Figure 5. Percent of *Aplysia* individuals laying eggs during the late SUMMER 1990. All animals were maintained in warm (20°C) water. Panel A: Intact, control animals were kept on short (8 h light/day) or long (16 h light/day) days. Data were averaged (+sem) into 5-day bins. Panel B: Bilaterally enucleated animals were kept on short (8 h light/day) or long (16 h light/day) days. Data are represented as in panel A. Panel C: Data from the 4 groups are presented as the percent of animals laying eggs each day, averaged (+sem) over the entire 30-day study. The letters a and b indicate values that are significantly different ($P < 0.05$). *Indicates that values approached significant difference compared to that of the intact, long-day control group ($P < 0.10$).

A common phenomenon among some seasonally breeding vertebrates is a spontaneous shutdown of the reproductive system during the non-breeding season (lizard: Cueller and Cueller, 1977; birds: Hamner, 1967; Robinson and Follett, 1982; mammal: Robinson and Karsch, 1984). During this period of reproductive quiescence, previously inductive photoperiodic cues no longer stimulate reproductive activity. This period of insensitivity to stimulatory photoperiod (commonly labelled 'photorefractoriness') is an endogenous process and can be 'broken' by exposing the animal to a bout of inhibitory photoperiod, followed by a stimulatory day length (Jackson *et al.*, 1988). In *Aplysia*, we have shown that previously stimulatory environmental cues (warm water, short days) did not stimulate spontaneous egg laying during the non-breeding season in winter and spring. *Aplysia* may therefore behave like many other seasonal breeders and become refractory to stimulatory signals. If this is so, then pretreatment with long days and cold temperatures should be able to break refractoriness to stimulatory short days and warm temperatures.

But mechanisms other than an endogenous refractoriness to an environmental signal might equally well underlie the cessation of spontaneous egg laying by *Aplysia* during winter and spring. For instance, one or more key components of the reproductive neural axis may be developmentally immature during the winter and spring (even though the reproductive tract can mature in the laboratory). Alternatively, some environmental cue (*e.g.*, food or other nutritional item necessary for high levels of spontaneous egg laying) may be missing during that time of year.

Further, our results suggest that the eyes play a role in mediating photoperiodic information to the reproductive axis responsible for regulating egg laying behavior. Specifically, photoperiod had no effect on egg laying in those animals that were bilaterally enucleated. The eyes of *Aplysia* contain both photoreceptors and a circadian pacemaker (Jacklet, 1969; Eskin, 1971). The circadian system is involved in the neural pathway mediating photoperiodic responses in most animals investigated (Follett and Sharp, 1969; Elliott, 1976; Almeida and Lincoln, 1982). Furthermore, both ocular and extraocular photoreceptors mediate photoperiodic responses in a variety of species (Reiter, 1969; Follett *et al.*, 1975; Legan and Karsch, 1983; Foster and Follett, 1985). In *Aplysia*, photoreceptors are found not only in the eye, but also in structures as diverse as the abdominal ganglion (Andresen and Brown, 1982), the cerebral ganglion (Block and Smith, 1973), the rhinophores (Jacklet, 1980), the oral veil (Cook *et al.*, 1991), and the siphon (Lukowiak and Jacklet, 1972). Therefore, the relative roles of the ocular photoreceptors and ocular pacemakers in mediating the effects of photoperiod on egg laying are not clear. For instance, both the ocular photoreceptors and ocular pacemakers may be playing a role in photoperiodic time measurement. Alternatively, extraocular photoreceptors may be transmitting light signals to the ocular circadian pacemaker, which then sends its signals to the next step in the photoperiodic response system.

Nevertheless, we must stress that our results are difficult to interpret, because the photoperiodic response in the intact controls in the last experiment was weak compared to that seen in the first experiment (compare Fig. 1 with

Fig. 5). An intriguing mystery arising from these studies is the source of the variability in the photoperiodic response from one year to the next. Because we work with animals captured in the wild, we have no control over the environmental history of the animal. That is, we cannot control for variations in microhabitat (*i.e.*, local and year-to-year variability in water temperature, food availability, sexual experience). Large, year-to-year fluctuations in the availability of the algae that *Aplysia* feed upon were observed during the course of these experiments: algae were abundant in the summer to early autumn of 1988, but scarce in the summer to early autumn of 1989 and 1990 (Dan Stark, Alacrity Marine Supply, pers. comm.); and these fluctuations were associated with similar changes in the robustness of the photoperiodic response. In addition to photoperiodic and temperature signals, there are other environmental variables (*e.g.*, food or nutritional cues) that might affect spontaneous egg laying. All of these environmental cues may act in combination such that one variable alters the effectiveness of the other variables on the frequency of egg laying. For instance, the photoperiodic response during the breeding season may be more robust in those animals with a high level of nutrition; low nutrition may weaken the photoperiodic response. Food availability has pronounced effects on the photoperiodic diapause response in some insect species (Saunders, 1979). Future work investigating the role of food cues may provide some information on its importance in the expression of a robust photoperiodic response in *Aplysia*.

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